In the Specification: A substitute specification follows. A Marked-Up version of the substitute specification follows this.

Patent Application of

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for

TITLE OF INVENTION:

METHOD FOR THE MEASUREMENT OF BIOLOGICAL LIGAND BINDING BY DETECTION AFTER SECONDARY IMMOBILIZATION.

CROSS REFERENCE TO RELATED APPLICATIONS: None

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT: This invention was not directly supported by any federally sponsored research.

REFERENCE TO SEQUENCE LISTING, TABLES, OR COMPUTER PROGRAM LISTINGS: None

BACKGROUND OF THE INVENTION

Frequently, researchers desire to analyze the ability of various proteins and other factors to bind to cell surfaces. Usually, the type of binding studied is one where the binding factor (ligand) recognizes and binds to a specific receptor for it on the cell surface. Thus, these types of studies are used to examine the inherent properties of the ligand itself, but also are used solely to study the receptor. Analysis of ligand binding to cell surfaces is usually performed directly, wherein that ligand itself is obtained in pure form and is radiolabeled. Usually, ligands are radiolabeled with ^{125}I. More rarely, they are purchased labeled with ^{3}H or ^{14}C. The labeled ligand is assessed for its maintenance of activity, and for its specific (cpm per unit of weight) radioactivity. To measure binding, the radiolabeled material is applied under established optimal conditions to desired cells of known density (cells/unit volume or cell protein/unit volume). Typically, various concentrations (from high to low) of the ligand are added to separate tubes or dishes of cells. Certain cell containers at each dose tested also receive an excess of unlabeled pure ligand. Usually, these excesses are 10 – 200 fold times the concentration of labeled ligand. After the desired binding time has passed, the unbound material from all samples is saved and the cells are washed free of all unbound labeled and unlabeled ligand. The cells are then placed into counting tubes and counted for radioactivity. Initial unbound material is counted also. The amount of labeled ligand bound or unbound is calculated from the known specific cpm. Counts obtained from unlabeled excess ligand-receiving samples are subtracted from the counts obtained from samples treated with like-dose labeled ligand only. This provides specific cpm bound. The weight

amount of specific labeled ligand bound is calculated from the known specific cpm per unit weight. Knowing the cell density, one can calculate amount of specific ligand bound per cell at each ligand dose level. Usually, the data is plotted as specific ligand bound/ ligand unbound/ unit of cells on the y axis and specific ligand bound/unit of cells on the x axis. This produces data with a negative slope and the x-intercept is the maximum amount of ligand able to bind. Therefore, the x-intercept also represents the receptors/cell for the ligand. This type of analysis is referred to as a Scatchard analysis. (Inoue *et. al.*, 1993; LaGrange *et. al.*, 1993; Schaffer, 1994; Gordon, 1995; Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999;).

An alternative method to determine ligand binding to cells is to conjugate a particular fluorescent molecule to the pure ligand. Fluorescent labeled material is allowed to bind to cells at various concentrations with or without the presence of unlabeled ligand. After binding is complete, all unbound ligand is washed off and the fluorescence of the cells is determined using a fluorescent spectrophotometer or a fluorescent activated cell sorter instrument (Gordon, 1995; Niedergang *et. al.*, 2000; Palupi *et. al.*, 2000, US patents 5,576,436, 5,962,223, 6,027,890, 6,815,212). This procedure is more difficult to standardize and precise quantitation of ligand receptors/cell is not as accurate as with Scatchard analysis using radiolabeled ligand. This method is more given to comparing binding capacity between two different cell populations. With fluorescent activated cell sorting, it also requires that the binding surface exist in a monodispersed state capable of being analyzed in the flow cell of that instrument.

It is also possible to allow ligand binding to cell surfaces and to then incubate the cells with a fluorescent labeled antibody to the ligand, wash, and analyze cell fluorescence by fluorescent spectrophotometry or fluorescent activated cell sorting. To assess ligand receptor levels only, one can incubate cells with a fluorescent labeled antibody to the receptor and measure the fluorescence of the cells by fluorescent activated cell sorting (Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999).

Western blotting is a technique where cell lysates obtained by detergent treatment are separated by electrophoresis and the separated components contained within the electrophoresis gel are driven onto a protein-binding membrane via electric current. The membrane with its cell constituents separated by molecular weight is blocked with a non-specific protein and can than be analyzed for particular cellular constituents by treatment with an antibody to that constituent followed by treatment with an enzyme conjugated antibody to the first antibody. Enzyme containing regions of the membrane are detected using color-producing or light-emitting substrates for that enzyme.

Dot-blotting or slot-blotting is where the cell lysate is applied directly to a binding-membrane without prior separation by electrophoresis. The membrane is blocked and treated as described in the previous paragraph to detect particular cell constituents. Unlike Western-blotting, the molecular weight of detected material is not ascertained.

We found that the binding of transferrin to tumor cell surfaces correlated with the aggressiveness of those cells; i.e.: the more metastatic tumor cells bound more transferrin than did poorly metastatic cells (Cavanaugh and Nicolson, 1991, Cavanaugh and Nicolson, 1998; Cavanaugh *et. al.*, 1999). These studies required that we accurately

assess the transferrin binding capability of cells in question. Initially, this was performed by examining the ability of the cells to bind ^{125}I-transferrin and the ability of non-labeled transferrin to inhibit that. Dealing with radioactive iodine has many drawbacks including the inherent hazardous nature of the material, its short shelf life, and expensive waste disposal. In searching for novel methods for measuring transferrin binding using non-radioactive procedures, we came upon the discovery that fluorescein-labeled transferrin would stimulate the growth of cells in culture similarly to native transferrin. We also found that fluorescein-labeled transferrin could be internalized by cells and that this internalization could be competed for by an excess of un-labeled (or native) transferrin. The apparent retention of biological activity by fluorescein-labeled transferrin lent us to examine other technologies available to specifically detect the labeled protein. Many antibody suppliers now sell anti-fluorescein antibodies. These were initially developed to detect fluorescein-labeled oligonucleotides hybridized to sample RNA on Northern blots. These same antibodies can easily detect fluorescein-labeled proteins on Western blots (Samuel *et. al.*; 1988, Haselbeck, *et al.*, 1990, Haselbeck and Hosel, 1992). We next assessed as to whether or not the combination of these reagents together would allow for the detection of fluorescein-labeled protein bound to cell surfaces. Cells were treated with fluorescein-labeled transferrin with and without an excess of native transferrin. After an appropriate incubation period, the cells were washed extensively and lysed with a detergent containing buffer. The lysate components were separated by electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was blocked with non-fat dry milk and incubated with a rabbit anti-fluorescein antibody. The membrane

was washed and incubated with goat horse radish peroxidase-conjugated anti-rabbit IgG. The membrane was washed again and treated with a light emitting (enhanced luminescence) substrate for horse radish peroxidase. One band at ≈70,000 in molecular weight was seen in all lanes loaded with cells that initially were exposed to fluorescein-labeled transferrin only. In lanes loaded with cells that had also received an excess of native transferrin, a markedly reduced band, or no band at all was seen. This method allowed for the sensitive determination of transferrin binding to cells without the need for radioactively labeled transferrin. Furthermore, the molecular weight of the bound ligand was verified via the electrophoresis step.

The major difference in the method of this ligand binding method in comparison to those of the referenced patents and literature papers is the final detection method. In our case, the bound hapten-ligand is detected by immunological means after solubilization (or cell lysis) and immobilization onto a membrane. In the referenced cases, bound radio-labeled ligand is detected on solubilized cells by counting; or in the case of fluorescent-labeled ligands, by fluorescent detection of the label on intact cells by optical means such as cytometry.

BRIEF SUMMARY OF THE INVENTION:

The present invention relates to the need in biological research to measure the ability of cells or other surfaces to bind a given compound (hereafter referred to as a ligand). The ligand could be a growth factor or any other factor whose study involves the need for persons to assess the ability of cells, or any other insoluble particle or material, to bind it. The invention requires that the binding factor be conjugated with an immunogical reactive hapten such as fluorescein and at the same time retain biological and binding activity.

The invention makes use of many available anti-hapten antibodies which specifically recognize a hapten-conjugated binding entity or ligand (hapten-ligand) in a complex mixture of other compounds which are naturally devoid of the hapten. The hapten-ligand is presented in excess to the substrate to which it binds. After binding, excess hapten-ligand is washed off, and all bound hapten-ligand is solubilized with or without solubilized substrate components. The solubilized mixture is applied to a membrane support directly or is separated by electrophoresis and then applied to a membrane support. The included membrane-bound hapten-ligand is detected by treatment of the membrane with anti-hapten antibody and then by an enzyme-conjugated-antibody to the anti-hapten antibody. The amount of resultant membrane-associated localized enzyme is determined by incubation with a color or light-producing substrate for that enzyme. For maximum sensitivity, a light-producing substrate is applied and the enzyme is detected by enhanced chemi-luminescence. A series of known amounts of pure hapten-ligand can

be applied to the membrane support, or can be separated by electrophoresis and applied to the membrane support, and similarly detected, to determine a signal to dose standard curve which can be used to ascertain the amount of hapten-ligand in the unknowns. Thus, the system lends itself to very precise and user-defined standardization. The two-antibody incubation steps amplify the signal so that in combination with enhanced chemi-luminescence, very low levels of hapten-ligand can be detected. When used this way, the system can be used to measure ligand binding to cell surfaces without the need for radio-labeled ligand. Another feature of the system is that all of the reagents required are stable and have long shelf-lifes. The system is a low-cost, non-hazardous, sensitive, non-radioactive, precisely standardized method for determining the binding of compounds to substrates. In particular, the method lends itself to the measurement of hapten-conjugated protein binding to cell and tissue surfaces. Specifically, the method has been perfected for the use of measuring fluorescein-conjugated transferrin, fluorescein-conjugated concanavalin A, fluorescein-conjugated annexin-V, fluorescein-conjugated avidin, and fluorescein-conjugated insulin binding to tissue culture cell surfaces. This invention not only offers a novel non-radioactive method for assessing ligand binding to cell surfaces, but can be used to quantitate the binding of any recognizable hapten-containing binding factor to any surface, providing that the factor can be subsequently removed, (and perhaps separated by electrophoresis; optional), and bound to a membrane support.

BRIEF DESCRIPTION OF THE DRAWINGS:

Figures 1A-1N show a schematic of the strategy of the assay, illustrating the measurement of the binding of fluorescein labeled transferrin to cell surfaces.

Figures 2A-2F show reproductions of actual enhanced chemiluminescence films of electrophoretically separated unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein labeled transferrin to cell surfaces.

Figures 3A-3N show a schematic of the strategy of the assay, illustrating the measurement of cellular apoptosis by analyzing the binding of fluorescein labeled annexin-V to cell surfaces.

Figures 4A-4C show a reproduction of the actual enhanced chemiluminescence film of electrophoretically separated unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein annexin-V to cell surfaces.

Figures 5A-5C show a reproduction of the actual enhanced chemiluminescence film of unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein conjugated concanavalin A to cell surfaces, after separation by electrophoresis.

Figures 6A-6D show a reproduction of the actual enhanced chemiluminescence films of unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein conjugated concanavalin A to cell surfaces by dot blotting, without preliminary separation.

Figures 7A-7C show a reproduction of the actual enhanced chemiluminescence film of electrophoretically separated unknowns and standards, and the graphical analysis of the luminescence data, obtained when analyzing the binding of fluorescein avidin to cell surfaces.

Figure 8 shows a reproduction of the actual enhanced chemiluminescence film of electrophoretically separated unknowns and standards obtained when analyzing the binding of fluorescein insulin to cell surfaces.

DETAILED DESCRIPTION OF THE INVENTION:

The object of the present invention is to provide a method for the sensitive non-radioactive assessment of ligand binding to insoluble surfaces. Specifically, the method developed measures the binding of transferrin, concanavalin-A, avidin, annexin-V, and insulin to cell surfaces.

A schematic of the detection and competitive binding strategy of the assay is shown in Figure 1. In Figure1A, a cell monolayer is exposed to a solution of fluorescein labeled transferrin (FITC-Tf). In Figure 1B, an identical cell monolayer is exposed to a solution of FITC-Tf plus an excess of unlabeled transferrin. In either case, 3 molecules of Tf bind per cell. When washed (Figure 1C) and lysed, cells from Figure 1A produced a lysate containing 9 molecules of FITC-TF (Figure 1E), whereas cells from Figure 1B produce a lysate containing 1 molecule of FITC-TF (Figure 1D, Figure 1F). In Figure 1G, both samples are loaded onto an electrophoresis gel, along with standards containing increasing levels of known amounts of FITC-Tf. When electrophoresed, the Tf and cell lysate proteins stack up and migrate according to their molecular weight (Figure 1H). These are blotted onto a membrane as shown in Figure 1I, where their relative positions are maintained. The membrane is blocked and treated with goat anti-FITC (Figure 1J), which specifically binds to the FITC-Tf only. The membrane is washed and treated with anti-goat IgG-peroxidase, which recognizes only the goat anti-FITC (Figure 1K). The membrane is washed again and treated with an enhanced chemiluminescent (ECL) substrate for peroxidase (Figure 1L), where light produced per band correlates with

membrane FITC-Tf content per band. The light produced is recorded on an X-ray film (Figure 1M), which is imaged so that each band is assessed for optical density/mm^2 (ODu/mm^2; Figure 1N). With the cells from Figure1A, a large band is seen on the X-ray film exposed by this light, at the same vertical position as the standard signals. Thus, the detection of the ligand is seen, at the correct molecular weight for transferrin. The amount of FITC-Tf in this band can be estimated by comparing its signal to that of the standards. The amount of FITC-Tf bound per cell is then calculated, from the cell density of the culture plate used in Figure 1A. When the FITC-Tf band produced from the cells from Figure1B is analyzed, minimal light production is seen. Thus, competition for Tf binding to the cells between FITC-Tf and Tf is seen, demonstrating specific binding to the cells by the FITC-Tf.

The basic detailed method using fluorescein-conjugated transferrin as a detectable antibody-recognizable hapten tracer follows:

Fluorescein-conjugated iron-saturated (holo) human transferrin was obtained from commercial sources. Cultured cells to be measured were grown to 50 – 60 % confluence in 12 well plates. Cells were incubated with serum-free minimal essential media (alpha modification; ∝-MEM) for 12 h and then again with fresh ∝-MEM for another 12 h. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. Media in remaining wells was replaced with 1 ml binding buffer (BB) which consisted of: 25 mM HEPES in ∝-MEM containing 2mg/ml of bovine serum albumin (BSA); pH 7.5. The cell wells were then allowed to equilibrate to 4°C in

a refrigerator. Sets of 5 replicate wells received increasing amounts of FITC-Tf, from 0.06 to 2.0 μg/ml final FITC-Tf. Two wells of each FITC-Tf concentration set then received unlabeled holo human transferrin so that the final unlabeled Tf concentration was 100 μg/ml. After a 2h incubation at 4°C, all media was saved (= unbound samples), and the wells were all washed 4 times by the addition and drainage of 1 ml of 4°C phosphate-buffered saline (PBS). All wells then received 0.5 ml of an RIPA cell lysing solution which consisted of PBS containing 1% v/v nonidet P-40 detergent, 0.5% v/v deoxycholic acid, 0.1% v/v sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethyl sulfonyl chloride, and 0.1 units/ml Aprotinin. Cells were incubated with the lysing solution for 30 min at 4°C and all lysates were pipetted into separate 1.5 ml conical tubes. The tubes were centrifuged at 5,000 X g for 10 min and 400 μL of each supernatant was transferred to a fresh tube. All of these tubes received 166 μL of a 4X concentrate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) treatment solution (0.5 M Tris, 8% w/v SDS, 2% v/v beta--mercaptoethanol, 1.0% w/v bromophenol blue, 20% v/v glycerol, pH 6.8), and were treated at 95°C for 10 minutes. Treated samples (150 uL each) were loaded onto an acrylamide SDS-PAGE electrophoresis gel. The gels consisted of a 12 X 10 cm separating gel containing 0.375 M Tris, 0.1% w/v SDS, 10 % w/v acrylamide, pH 8.8; and a 12 X 2 cm stacking gel containing 0.125 M Tris, 0.1% w/v SDS, 4% w/v acrylamide, pH 6.8.

Unbound samples were treated similarly to cell lysate samples, and loaded onto said electrophoresis gel. Typically, these have to be diluted 1:10 – 1:100 in 1 X SDS-PAGE

treatment solution, prior to electrophoresis, to produce a signal within a readable range. The assay was standardized by loading a series of treated solutions of known amounts of pure FITC-Tf onto said electrophoresis gel. These consisted of 8 samples applied so that 2.4, 4.8, 9.7, 19, 39, 78, 156, and 313 ηg FITC-Tf protein were delivered per well, respectively. All samples were electrophoresed at 40 mA constant current until the dye front was 1 cm from the bottom of the gel. The gel was equilibrated in a transfer buffer of 48 mM Tris, 39 mM glycine. A 14 X 14 cm nitrocellulose membrane was equilibrated in transfer buffer and the gel and membrane assembled into a transfer apparatus and immersed in transfer buffer. Gel components were transferred to the membrane at a constant voltage of 40 V for 1.5h.

The membrane was blocked at 4°C overnight in a block solution consisting of Tris buffered saline (TBS: 25 mM Tris, 0.15 M NaCl, pH 7.8) containing 0.1% tween 20 and 5% w/v non-fat dry milk. The membrane was incubated with 1:1000 rabbit anti-FITC in block solution for 2h at 25°C, and washed three times (20 min each) with 50 ml TBS. The membrane was incubated with 1:2000 horse radish peroxidase-conjugated goat anti-rabbit IgG in block solution for 2h at 25°C and washed again. The membrane was covered with an enhanced chemiluminescent substrate for horse radish peroxidase, was wrapped in plastic, and was loaded into an X-ray film cassette along with an 8 X 10 inch piece of chemiluminescent-detecting film. The film was developed after 1 min exposure and an additional film was added which was developed after 20 min exposure. Bands

produced on the film were quantitated by measurement of total optical density/mm^2, using a charged coupled device (CCD) camera equipped imager.

Figure 2A displays an image of the film from the transferrin binding method obtained after the 1 min exposure. Here, all lanes were loaded with lysates from equal quantities of cells initially exposed to the concentration of FITC-TF listed above the blot. Signals from duplicate wells are shown. The figure shows that when increasing levels of FITC-Tf are initially present, that higher levels of FITC-Tf bind to a constant amount of cells, which is in keeping with normal binding behavior. In Figure 2B, the left half of the gel was loaded with cells initially treated as in Figure 2A, but also with 100 μg/ml of unconjugated Tf. The figure also displays markedly lower binding of FITC-Tf to the cells when an excess of Tf is initially present, indicating competition between FITC-Tf and Tf for cell binding, and therefore specific binding of FITC-Tf to the cells. The right half of Figure 2B shows results from the analysis of equal amounts of aliquots of the initial unbound samples from Figure 2A.

Figure 2C shows an image of the film obtained when standard solutions of FITC-Tf were electrophoresed, blotted, and analyzed for FITC content as indicated above. Here, the amounts of FITC-Tf loaded onto the electrophoresis gel (in ηg) are indicated on the top. Figure 2D displays the standard curve obtained when the density of the bands obtained from the image analysis of the film from Figure 2C were plotted against the amount of FITC-Tf present in each band. The equation shown on the curve was used to calculate the amount of FITC-Tf present in the bands from Figure 2A and 2B, thus enabling the determination of the weight of FITC-Tf bound per cell, and unbound per

well, at each initial FITC-Tf concentration. This data was converted to molecules and moles, using a Tf molecular weight of 75,000. This data was then plotted as a conventional Scatchard analysis as shown in Figure 2F, to obtain Tf receptors per cell.

The binding of annexin V to cell surfaces has been recognized as an indicator of early apoptosis (Zhang *et. al.*, 1997). With conventional procedures, cells are removed from plates, treated with FITC-annexin V, and analyzed by FACS. The removal of cells from tissue culture plates using conventional trypsin or EDTA reagents can in itself induce cell stress, apoptosis, and cellular annexin V binding (LeGall *et. al.*, 2000). Therefore, the conventional use of annexin V binding as a measure of apoptosis in adherent cells is problematic. In contrast, this new method would measure the binding of FITC-annexin V to adherent cultured cells *in situ* (Figures 3 and 4), where binding and washing occur first, before the cells are removed from plates for analysis. Therefore, the amount of FITC-annexin V detected would accurately represent that bound by cells in their natural culture environment. Thus, the method outlined in this new method circumvents conventional problems and provides for a more authentic measure of natural cellular annexin V binding.

A schematic of the strategy of the assay when used to detect apoptotic cells is shown in Figure 3. Cells in apoptosis (Figure 3A) or normal non-apoptotic cells (Figure 3B) are exposed to a solution of FITC-Annexin V. When washed (Figure 3C) and lysed, cells from Figure 3A produced a lysate containing FITC-Annexin V (Figure 3E), whereas cells from Figure 3B produce a lysate containing no FITC-Annexin V (Figure 3D, Figure 3F). In Figure 3G, both samples are loaded onto an electrophoresis gel, along with standards

containing increasing levels of known amounts of FITC-Annexin V. When electrophoresed, the Annexin V and cell lysate proteins stack up and migrate according to their molecular weight (Figure 1 H). These are blotted onto a membrane as shown in Figure 3I, where their relative positions are maintained. The membrane is blocked and treated with goat anti-FITC (Figure 3J). This specifically binds to the FITC-Annexin V only. The membrane is washed and treated with peroxidase-anti-goat IgG, which recognizes only the goat anti-FITC (Figure 3K). The membrane is washed again and treated with an ECL substrate for peroxidase (Figure 3L), where light produced per band correlates with membrane FITC-Annexin V quantity per band. The light produced is recorded on an X-ray film (Figure 3M), which is imaged so that each band is assessed for ODu/mm^2 (Figure 3N). With the cells from Figure 3A, plentiful FITC-Annexin V binds, this is then present on the blot, the initial antibody and therefore the second antibody bind, light is produced upon incubation with an HRP chemiluminescent substrate, and a band is seen on the film. The amount of FITC-Annexin V in this band can be estimated by comparing its signal to that of the standards. The amount of FITC-Annexin V bound per cell is calculated, from the cell density of the culture plate used in Figure 3A. With the cells from Figure 3B, no FITC-Annexin V binds, none is present on the blot, the initial antibody and therefore the second antibody do not bind, no light is produced upon incubation with an HRP chemiluminescent substrate, and no band is seen on the film.

The detailed methods for the lysis, electrophoresis, blotting, and ECL detection steps for annexin V, concanavalin A, Avidin, and insulin binding assays were the same as those outlined above in detail, for transferrin. The other specifics of these assays follow.

Figure 4 displays actual results obtained when this assay was used to measure the binding of FITC-Annexin-V to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. Cells were induced to apoptose by treatment with 4 μg/ml Camptothecin (dissolved in DMSO). Controls received DMSO only. After 24 hours, wells were washed three times with and equilibrated in 1 ml binding buffer (25 mM HEPES, 0.15 M NaCl, 2.5 mM $CaCl_2$, pH 7.5). FITC-Annexin V was added to 50 ηg/ml and the cells were incubated for 30 min at 25° C. Cells were then washed extensively with binding buffer, and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., the supernatants were assessed for total protein, and equal protein equivalents of the supernatants were treated (by the addition of one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) for, and separated by SDS-PAGE. Also run on the same gel were four pure FITC-Annexin V standards of 0.5, 1, 2, and 4 μg Annexin V protein per well. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and then incubated with rabbit anti-FITC, and then with goat anti-rabbit IgG-HRP. HRP containing bands were then detected by ECL. Figure 4A shows a scan of the ECL detection film, with each lane marked at the top as to the sample applied. Figure 4B displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 4A were plotted against the amount of Annexin-V present in each standard band. The equation shown on the curve was used to calculate the amount of Annexin-V

present in the bands from the cell lysates, thus enabling the determination of Annexin-V bound per cell equivalent (or cell protein) for the various treatments (Figure 4C).

To further test and illustrate another embodiment of the assay, the ability of the method to detect the binding of Concanavalin A (Con A) to cells was examined. Figure 5 displays the results obtained when this assay was used to measure the binding of FITC-Con A to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in replicate, in six well plates. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. The growth media of test wells was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Con A was added to replicate wells so that the final concentrations of FITC-Con A were 0.1, 1.0, and 10.0 μg/ml. One well of each FITC-Con A concentration also received 200 μg/ml of native (un-conjugated Con A). The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 800 uL of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and equal cell equivalents of the lysate supernatants were treated for (by the addition of one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) and separated by SDS-PAGE. Also run on the same gel were four pure FITC-Con A standards consisting of 1, 2, 4, and 8 ηg total FITC-Con A protein loaded per lane, respectively. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP.

HRP containing bands were detected by ECL onto an X-ray film. The film was imaged to obtain the optical density units/mm^2 of the bands. A scan of the film is shown in Figure 5A. Figure 5B displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 5A were plotted against the amount of FITC-Con A present in each standard band. The equation shown on the curve was used to calculate the amount of Con A present in the bands from the cell lysates, thus enabling the determination of Con A bound per cell equivalent for the various treatments, as shown in Figure 5C. Cells which were initially treated with both FITC-Con A and un-conjugated Con A displayed markedly lower binding of FITC-Con A than cells which received FITC-Con A only, indicating competition for binding between FITC-Con A and unconjugated Con A, further indicating specific cell binding by the FITC-Con A.

The replacement of electrophoresis with dot-blot techniques is possible. This would require that the only immune-recognizable conjugated component present prior to dot-blotting would be the desired product and/or absolutely minimal interaction of either antibody with non-specific sample components

Figure 6 displays the results obtained when the samples from Figure 6 were analyzed by a dot blot procedure. For the standards, increasing volumes (2, 4, 8, and 16 μL) of a 100 ηg/ml FITC-Con A solution were applied to a nitrocellulose membrane. For the lysates, 4 μL of lysates from cells treated with 0.1, 0.5, and 1.0 μg/ml FITC Con A (with or without an excess of native Con A) were applied to the membrane. The membrane

was blocked, incubated with rabbit anti-FITC, then with goat anti-rabbit IgG-HRP, and HRP-containing sites detected with ECL onto an X-ray film. The scans of the actual films from the standards are shown in Figure 6A, and that for the cell lysates in Figure 6B. The dots on the film were quantitated using an imager to obtain the optical density units/mm^2 of each dot. Figure 6C displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 6A were plotted against the amount of Con A present in each standard dot. To maintain linearity, only the first three were used. The equation shown on the curve in Figure 6C was used to calculate the amount of Con A present in the dots from the cell lysates in Figure 6B, thus enabling the determination of Con A bound per cell equivalent for the various treatments, as shown in Figure 6D. As with Figure 5, cells which were initially treated with both FITC-Con A and un-conjugated Con A displayed markedly lower binding of FITC-Con A than cells which received FITC-Con A only, indicating competition for binding between FITC-Con A and unconjugated Con A, further indicating specific cell binding by the FITC-Con A. This displays the usefulness of the technique in a dot-blot procedure, where the SDS-PAGE and electroblotting steps are eliminated.

Another experiment designed to test and illustrate the use of the method, was one where the examination of the ability of the method to detect the binding of avidin to cells was conducted. Figures 7A - 7C display the results obtained when this assay was used to measure the binding of FITC-Avidin to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter.

The growth media was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Avidin was added to replicate wells so that the final concentrations of FITC-Avidin were 0.1, 0.2, and 0.4 μg/ml. The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 1 ml of RIPA lysing solution/well. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants representing equal cell equivalents were treated (by the addition of one third volume 4X sample treatment solution and exposure to 95° C for 5 minutes) and separated by SDS-PAGE. Also run on the same gel were four pure FITC-Avidin standards of 100, 200, 400, and 800 ρg total FITC-Avidin protein loaded per lane, respectively. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL onto an X-ray film. The film was imaged to obtain the optical density/mm^2 (ODu/mm^2) of the bands. A scan of the film is shown in Figure 7A. Figure 7B displays the standard curve obtained when the density of the bands obtained from the image analysis of the standards from Figure 7A were plotted against the amount of FITC-Avidin present in each standard band. The equation shown on the curve was used to calculate the amount of FITC-Avidin present in the bands from the cell lysates, thus enabling the determination of FITC-Avidin bound per cell equivalent for the various treatments, as shown in Figure 7C.

To further test the versatility of the method, its ability to detect the cellular binding of the low molecular weight protein insulin was examined. Figure 8 displays the results obtained when this assay was used to measure the binding of FITC-Insulin to human K562 erythroleukemia cells. Logarithmically growing cells in suspension culture were collected by centrifugation and washed twice by suspension in and centrifugation from a binding buffer consisting of alpha-MEM containing 5 mg/ml BSA and 25 mM HEPES (pH 7.5). Cells were adjusted to a density of 2 X 10^6/ml (in binding buffer), and were equilibrated to 4° C. To 1 ml of cell suspension was added 20 µl of 1 mg/ml FITC-Insulin (in binding buffer; final concentration in cell suspension = 20 µg/ml). An additional tube also received non-conjugated Insulin at a level of 200 µg/ml. Cell suspensions were incubated for 2h at 4° C while rotating slowly, and the cells were collected and washed three times by suspension in and centrifugation from binding buffer. The initial supernatants were kept as the unbound samples. Cell pellets were lysed in 0.4 ml/tube of Schagger-Von Jagow (SVJ) electrophoresis system treatment solution (50 mM Tris-HCl, 2% w/v SDS , 1% v/v beta-mercaptoethanol, 5% v/v glycerol, 0.1% w/v bromophenol blue, pH 6.8), and treated at 95° C for 5 min. Unbound samples were likewise treated by the addition of one third volume of a 4X concentrate of the sample treatment solution, and exposure to 95° C for 5 min. Lysates (100 µl each) and aliquots of the treated unbound samples were separated by SDS-PAGE run according to Schagger-Von Jagow. These gels consisted of a 12 X 10 cm separating gel containing 1M Tris, 0.1% w/v SDS, 12% w/v acrylamide, pH 8.45; and a 12 X 2 cm stacking gel

containing 0.75M Tris, 0.1% w/v SDS, 4% w/v acrylamide, pH 8.45. Gels were run in an electrode buffer of 0.1M Tris, 0.1M Tricine, 0.1 % SDS. Also run on the same gel were three treated pure FITC-Insulin standards of 1, 2, and 4 ηg total FITC-Insulin protein loaded per lane, respectively. Gel components were transferred to a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then with goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL onto an X-ray film. In the Figure 8, a scan of the X-ray film is shown. The lanes of the membrane are shown at the top, and the samples that those lanes received are shown at the bottom. Increasing signal is returned for increasing loads of FITC-insulin in the standards (Figure 8, lanes 1-3). The FITC-insulin bound by the cells is easily observed (Figure 8, lane 4), and this is reduced significantly when excess un-conjugated insulin was present (Figure 8, lane 5). This procedure consistently displays higher molecular weight forms of insulin formed after application to cells, perhaps due to the presence of insulin binding proteins (Figure 8, lanes 4-7).

General applications: The assay strategy can apply to any ligand conjugated with a compound which can be specifically recognized by an antibody. In particular, anti-digoxygenin, anti-rhodamine and anti-biotin antibodies exist which would recognize ligands conjugated with those compounds. The material to which the ligand binds to can be other than cells. Any particles or other insoluble material can serve as the binding surface. Centrifugation and re-suspension of suspended particulate binding substrates would serve as a method for washing those of unbound ligand. The current method requires that the bound ligand be removed from the binding surface so that it can be

separated by electrophoresis. It must also bind to a conventional transfer membrane for detection with the antibody. Other specific applications accomplished to date include the study of the binding of FITC-conjugated concanavalin A to cells, the study of the binding of Avidin to cells, the study of the binding of Annexin V to cells, and the study of the binding of insulin to cells. With the Annexin V protein, this assay could be utilized to assess cellular apoptosis without the need for a FACS analyzer.

In another embodiment, the assay could be used to verify the hybridization of a known biotin-labeled DNA to a surface. After binding, the bound labeled DNA is released through heat de-naturation, is separated by agarose electrophoresis, electro-blotted to nytran, and is detected by incubation with species-x anti-biotin followed by incubation with anti-species-x IgG-HRP and ECL. The final result yields a major band at the expected bp size of the labeled DNA. As with the above stated protein procedures, the proper molecular weight of the desired product is verified by comparison to standards of the labeled DNA run on the same electrophoresis gel.

Conclusion: this new method is a procedure for measuring the binding of an entity (ligand) to a surface by using a hapten-conjugated version of the ligand (hapten-ligand), where the hapten is recognizable by an antibody. An excess of the hapten-ligand is presented to the binding surface and excess (unbound) hapten-ligand is washed off. Bound hapten-ligand is then solubilized (removed) and applied to a membrane support or separated by electrophoresis and applied to a membrane support. Known quantities of the hapten-ligand are also applied to a membrane support or separated by electrophoresis and applied to a membrane support. The membrane-bound hapten-ligand is detected by

application of an enzyme-conjugated antibody to the hapten; or by application of an antibody to the hapten followed by application of an enzyme-conjugated antibody to the anti-hapten antibody. The resultant membrane-associated enzyme is detected and quantitated by the application of a color or light-producing substrate which reacts with the enzyme. Results obtained from the standards are used to construct a standard curve which is then used to calculate the amount of hapten-ligand in the membrane areas corresponding to the unknowns. Thus, the amount of hapten-ligand originally bound to the surface can be determined. This assay method has the advantages of providing verification of the molecular weight of the binding substance (ligand) via the electrophoresis step. It eliminates the need for radioactive materials. The procedure provides for high sensitivity detection as the dual antibody incubation steps amplify the signal significantly. The procedure allows for easy standardization as different user-definable levels of a standard solution of the Hapten-ligand can be simultaneously applied to the electrophoresis gel or to the dot-blot or slot-blot membrane